Matters Arising Response

Developmental Cell

Distinct Polarity Cues Direct Taz/Yap and TGFβ Receptor Localization to Differentially Control TGFβ-Induced Smad Signaling

Highlights

- A direct response to the Nallet-Staub et al. (2015) Matters Arising is presented
- Cytoplasmic sequestration of Taz/Yap with TGFβ-activated Smads is recapitulated
- Basal TGFβ receptor polarization in epithelia is distinct from Hippo-TGFβ crosstalk
- A model of temporal events regulating epithelial TGFβ-Smad signaling is presented

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In Brief

Narimatsu et al. address the Nallet-Staub et al. (2015) Matters Arising. The authors provide evidence that that TGFβ receptors' basal recruitment occurs subsequent to cytoplasmic TAZ/YAP-mediated, Hippo-dependent suppression of Smad activity, suggesting that receptor sequestration and Hippo control of activated Smads are distinct events regulating TGFβ signaling in polarized epithelia.

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Distinct Polarity Cues Direct Taz/Yap and TGFβ Receptor Localization to Differentially Control TGFβ-Induced Smad Signaling

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SUMMARY

We and others have shown that the Hippo pathway effectors TAZ and YAP direct Smad activity to regulate TGFβ family-induced cellular responses in stem cell and cancer biology. In polarized epithelial cells we showed that the Crumbs complex promotes Hippo-dependent cytoplasmic TAZ/YAP localization that restricts TGFβ-induced Smad nuclear accumulation and activity. In this *Developmental Cell* issue, basal-lateral restriction of TGFβ receptors is proposed as the sole mechanism suppressing Smad signaling in epithelial cells. Here we show that basal recruitment of TGFβ receptors occurs subsequent to Hippo-dependent suppression of Smad activity by cytoplasmic TAZ/YAP. Our results demonstrate that receptor sequestration and Hippo control of activated Smads are distinct events regulating TGFβ signaling in polarized epithelia and raise interesting questions about the function of these pathways in controlling Smad signaling in development, homeostasis, and disease. This Matters Arising Response addresses the Nallet-Staub et al. (2015) Matters Arising, published concurrently in *Developmental Cell*.

INTRODUCTION

The TGFβ family is an archetypical example of a multifunctional signaling pathway in which biological output is dependent on context. For example, TGFβ family members have the potential to both stimulate and inhibit proliferation, function to suppress and promote tumorigenic events, and direct both stem cell renewal and stem cell differentiation (Massagué, 2012). An elegant example of context defining biological output is provided by human embryonic stem cells (hESCs), in which TGFβ-Smad signaling is required for maintenance of pluripotency but also specifies the mesendoderm lineage (Beyer et al., 2013). Our studies, directed toward understanding how contextual biology is manifested at the molecular level, led to our findings that the Hippo pathway effectors TAZ and YAP (TAZ/YAP) interact with Smads and control TGFβ-regulated Smad activity. Our work showed that TAZ and YAP mediate nuclear Smad signals (Varelas et al., 2008, 2010) and that complexes between TAZ/YAP and activated Smads control hESC pluripotency (Beyer et al., 2013).

The Hippo pathway is comprised of a core kinase network in which Mst1/2 activate Lats1/2, which in turn phosphorylate and inhibit the transcription co-factors TAZ and YAP. The pathway was first discovered as a key regulator of tissue size control and later was identified as a key mediator of contact inhibition, with cell density activating the pathway to suppress nuclear TAZ/YAP activity. We showed that, when mammary epithelial cells achieve high density, apical-basal polarization and ultimately the assembly of the Crumbs complex coordinates Hippo pathway activity to restrict nuclear TAZ/YAP localization and suppress Smad nuclear accumulation and signaling (Varelas et al., 2010). Importantly, we extensively documented that under these conditions TGFβ-dependent activation of Smads was unaffected, that interference with Hippo pathway activity reconstituted Smad signaling, and that in lower-density cultures, ectopic activation of the Hippo pathway sequestered TAZ/YAP and Smads in the cytoplasm. We also showed in the inner cell mass of the mouse blastocyst that the Hippo pathway inhibits the nuclear localization of phosphorylated Smad2. These results demonstrated that the Hippo pathway can restrain Smad signaling and that, in polarized epithelial systems, the Crumbs complex couples cell density cues to Hippo pathway activity and Smad signaling. The implicit conclusion from this work is that it is not cell density per se that regulates Hippo and Smad signaling, but rather it is polarity cues (i.e., Crumbs) that are required, and these signals are necessary to couple cell density to Hippo activation. Indeed, culturing cell lines at high density (HD) does not automatically equate to Hippo pathway activation, as many transformed cells exhibit nuclear TAZ/YAP regardless of density (e.g., MDA-MB-231 [Hiemer et al., 2014]) and TAZ/YAP escape from cell density control is of key importance in driving cancer.

In the accompanying Matters Arising manuscript, Nallet-Staub et al. specifically question our work that showed that the Crumbs complex couples cell density in polarized epithelial cells to Hippo-dependent cytoplasmic TAZ/YAP and restriction of Smad localization and activity (Varelas et al., 2010). They recapitulate previously published work (Murphy et al., 2004) to propose that in polarized epithelial cells, basolateral localization of TGFβ receptors in response to increasing cell density is the

sole mechanism that inhibits Smad signaling in response to apically delivered TGFβ. Here we comment on their data and conclusions and provide interesting new data that not only reaffirm the findings of Varelas et al. (2010) but also reveal a multi-step mechanism integrating epithelial polarity with the regulation of TGFβ signaling. We show that while Hippo pathway activation is an early event in polarizing epithelial cells that promotes cytoplasmic sequestration of TAZ/YAP and suppression of TGFβ-Smad activity, prolonged culture can lead to the basal restriction of TGFβ receptors, thus reducing Smad activation. These molecular events offer potential insight into the rapid dynamics of TGFβ signals in development and the mechanisms restricting TGFβ signaling in homeostatic tissues.

RESULTS AND DISCUSSION

Following protocol details and employing the appropriate controls when attempting to reproduce results is important, particularly when examining complex biological systems, as recently commented on (Hines et al., 2014). Although Nallet-Staub et al. examined Eph4 mammary epithelial cells, the major cell model employed in Varelas et al. (2010), we noticed that the methods they used did not match our published protocols. For example, they used split ratios to define cell density, employed different culture conditions (extended culture in low serum), and varied TGFβ stimulation between experiments (30 min to 24 hr). Based on their discrepant conclusions, we considered whether alternative culture conditions might affect how Eph4 cells respond to TGFβ. For this, we repeated the experiments from our original publication but used an extended time course in which HD Eph4 cells were cultured for 8, 24, 48, or 72 hr and then treated for 1 hr with or without apically delivered TGFβ. Parallel cultures of cells were then examined in three ways: (1) examined by immunofluorescence microscopy for Taz/Yap together with Smad2 (Figure 1A) or activated phospho-Smad2/3 (P-Smad2/3) (Figure 1B); (2) lysed to examine Smad2 activation by immunoblotting (Figure 1C); or (3) analyzed by RT-qPCR for expression of canonical TGFβ (*Smad7* and *Pai1*)- and Taz/Yap (*Cyr61*)-regulated target genes (Figure 1D). At 8 hr, we observed Smad2 activation and strong expression of canonical TGFβ and Taz/Yap target genes that was accompanied by nuclear Taz/Yap and robust nuclear accumulation of total and activated Smads. By 24 hr, cytoplasmic Taz/Yap was evident, and while TGFβ-induced Smad2 phosphorylation was robust, nuclear accumulation of total and activated Smads was reduced, as was TGFβ- and Taz/Yap-regulated target gene expression. Importantly, by 48 hr, Taz/Yap were strongly sequestered in the cytoplasm, and despite robust TGFβ-dependent Smad phosphorylation, Smad nuclear accumulation and target gene expression were both further suppressed when compared to 24 hr. In this regard, we note that while Nallet-Staub et al. argue that phosphorylated Smad3 is rapidly lost in HD Eph4 cells, their results are open to alternative interpretations (see Figure 5B in Nallet-Staub et al., 2015). Regardless, by 72 hr, we noted that Smad activation was reduced overall, which suggested that access to TGFβ receptors might be compromised. To examine this, we generated Eph4 cells stably expressing Clover-tagged TβRII, which revealed that the receptors were distributed to the apical and basal-lateral cell membrane at up to 48 hr of HD culture (Figure 1E), consistent with the robust activation of Smads observed over this time frame. However, by 72 hr, although some apical receptors were still evident, there was clear basolateral accumulation, consistent with prior work (Murphy et al., 2004) and the observations of Nallet-Staub et al. Collectively, these results confirm our conclusions that nuclear accumulation of activated Smads is restrained in polarized epithelial cells by Taz/Yap and suggest that receptor sequestration is a distinct mechanism controlling Smad activation.

Our prior work emphasized that when investigating crosstalk between Hippo and TGFβ signaling it is important to analyze Hippo pathway activity, Taz/Yap localization, and, in epithelial cells, polarity and Crumbs assembly. We were therefore intrigued by the results from the Nallet-Staub et al. studies on Eph4 cells cultured on Transwell membranes, in which basal stimulation of "dense" cells induced TGFβ signaling. Because Nallet-Staub et al. did not examine Taz/Yap regulation, or localization, we did so in the same culture system. Interestingly, we observed poor cytoplasmic sequestration of Taz/Yap in Eph4 cells grown at HD on Transwell membranes (Figure 2A), escape from contact inhibition, as evidenced by multilayered growth (Figure 2B), and some Smad2 activation when stimulated with basal TGFβ (Figure 2A). Given that this cell model displays poor regulation of Taz/Yap localization, we question its use to study cytoplasmic Hippo-TGFβ crosstalk.

These studies reveal that regulation of Hippo is dynamic and can be readily uncoupled from control of Taz/Yap, consistent with the emerging role of this pathway as an important sensor of cellular context that can modulate the cellular response to Smad and other signaling pathways. Our studies underscore that placing cells at HD does not automatically equate to inhibition of Taz/Yap (see Introduction). Moreover, even epithelial cell polarity cues can be uncoupled from Taz/Yap, as is the case in the trophectoderm of the developing mouse blastocyst, where nuclear Taz/Yap is key for fate specification (Alarcon, 2010). Therefore, it is critical to incorporate analysis of Taz/Yap localization in any study of Hippo-TGFβ pathway crosstalk. Taken together, our results support a model (Figure 2C) in which Hippo-dependent control of Smad signaling via the Crumbs complex is an early event during polarization of epithelial cells, which suppresses the nuclear accumulation of activated Smad complexes, and is followed by basal-lateral sequestration of receptors that prevents Smad activation by apical TGFβ. Notably, we showed that Hippo regulation of activated Smad localization is important in the inner cell mass of the blastocyst and likely the epiblast of the gastrulating mouse embryo (Varelas et al., 2010). Thus, it will be interesting to pursue future in vivo studies to define what role receptor localization plays in controlling TGFβ responsiveness of epithelia.

Our previous publication extensively characterized how cytoplasmic Taz/Yap regulates Smad signaling in Eph4, MCF12A cells, and early mouse embryos. In their work, Nallet-Staub et al. extend their analyses to a series of cell lines that we did not examine. The human HaCat keratinocyte model is interesting in this respect, as under the conditions used by Nallet-Staub et al., the cells appear to display robust activation of Smads in response to apically applied TGFβ, even when TAZ/YAP are cytoplasmic (see Figure 2A in Nallet-Staub et al., 2015). These cells have thus uncoupled Smad regulation from both Hippo

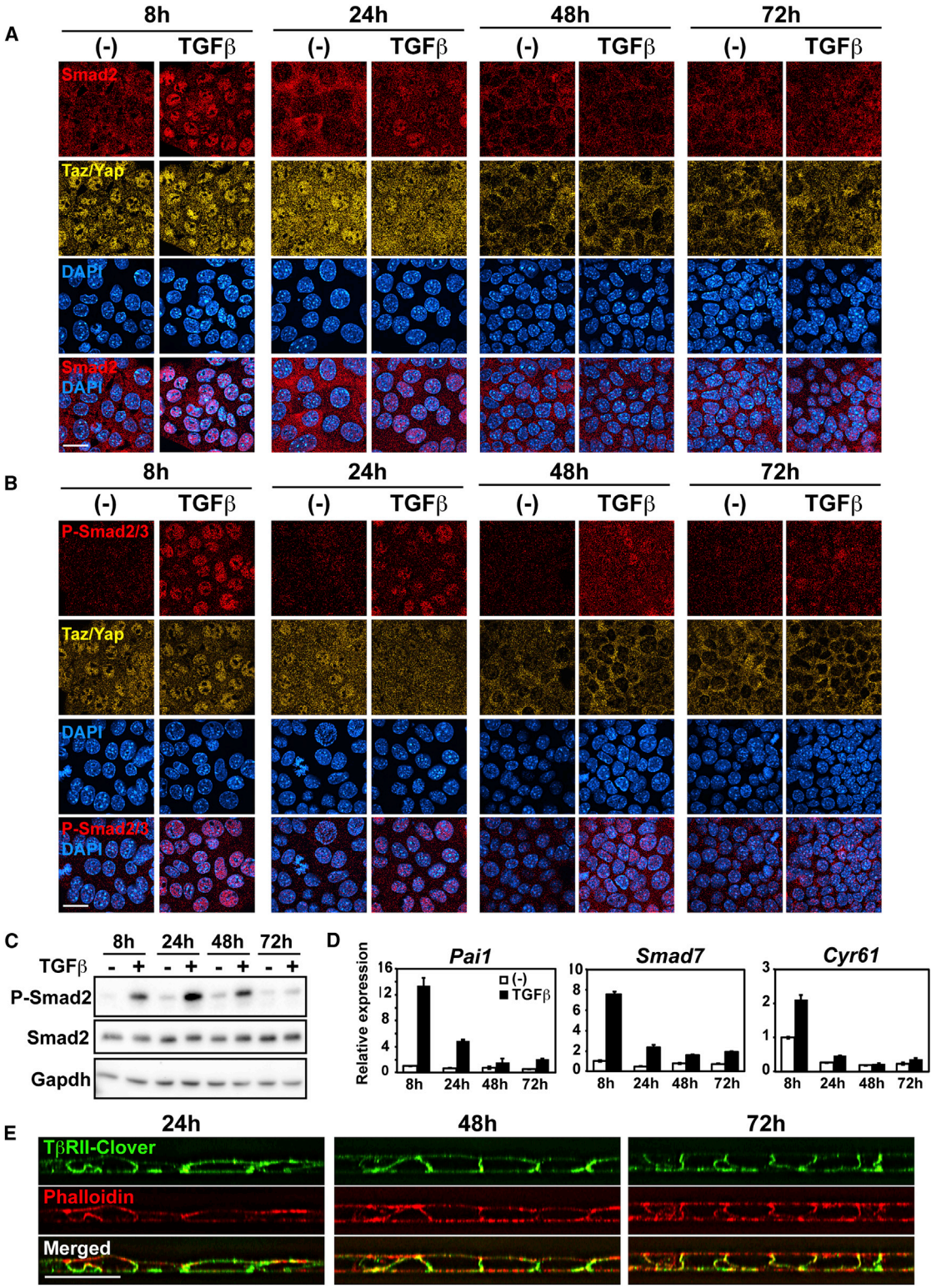

Figure 1. Temporal Distinction between Hippo-TGFβ Crosstalk and Receptor Sequestration in Eph4 Cells

Eph4 cells plated at HD, cultured for the indicated times, were stimulated with or without 100 pM TGFβ1 for 1 hr and then analyzed using various methods. Data shown are from a representative experiment that was performed three times independently.

(A and B) Immunofluorescence microscopy. Cells were fixed, and localization of (A) Taz/Yap and Smad2 or (B) Taz/Yap and P-Smad2/3 was visualized.

(C) Immunoblotting. Total and phosphorylated Smad2 levels were assessed in cell lysates.

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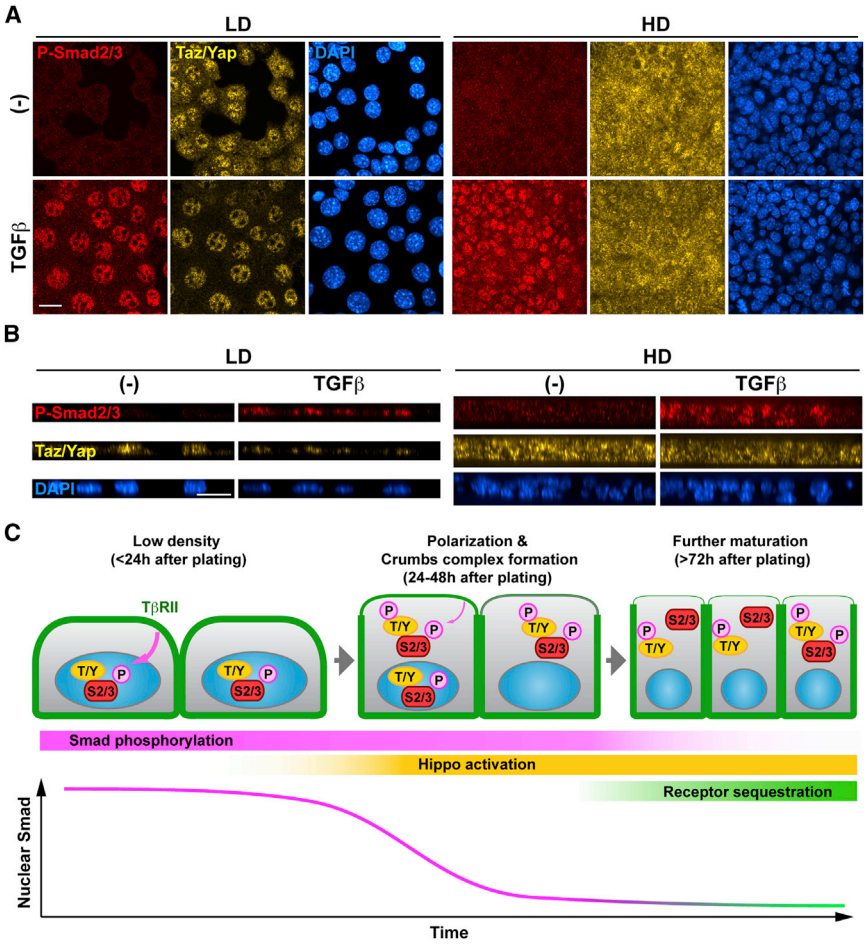

Figure 2. Smad and Taz/Yap Localization in Eph4 Cells Grown on Transwell Filters

Eph4 cells, plated at low or high density, were cultured on Transwell inserts for 48 hr and stimulated with 100 pM TGFβ for 1 hr from the basal side.

(A and B) (A) Optical XY plane images and (B) reconstructed cross-sections for phospho-Smad2/3, Taz/Yap, and nuclei are shown. Scale bars, 20 μm in all images.

(C) Model depicting distinct mechanisms by which cell polarity attenuates TGFβ-Smad signaling. In non-polar cells, Taz and Yap (T/Y) are nuclear localized, allowing efficient nuclear accumulation of phosphorylated Smad2/3 (S2/3). Polarity-mediated activation of Hippo signaling promotes sequestration of Taz/Yap in the cytoplasm, consequently restricting phosphorylated Smad2/3 from the nucleus. Maturation of epithelial polarity can further result in the basal localization of TGFβ receptors, attenuating Smad phosphorylation/activation when such cells are exposed to apical TGFβ.

crosstalk and receptor sequestration. As noted in the Introduction, the study of TGFβ biology provided some of the first examples of how biological responses to a single stimulus are strongly dependent on cell type and context. Thus, while our work clearly identified in vitro and in vivo models in which TGFβ-Hippo crosstalk provides context-dependent regulation of TGFβ response, it would be surprising if this extended to all aspects of TGFβ biology. Indeed, rather than a cause for alarm, understanding how and why specific systems bypass these controls on Smad signaling is an interesting area for further investigation.

Finally, while our work along with others (see below) has shown that Smads intersect with both TAZ and YAP, the role of YAP in TGFβ signaling was only considered by Nallet-Staub et al. in HaCat cells. They argue that there is no role for TAZ/YAP in TGFβ signaling, but no confirmation of functional TAZ/YAP knockdown by analysis of canonical target genes is provided, and they show limited analysis of knockdown efficiency (see Figure S3A in Nallet-Staub et al., 2015). Further, Nallet-Staub et al. only examined TAZ in other cell lines, similarly with no confirmation of functional knockdown. Regardless of whether TGFβ is coupled to the Hippo pathway in the transformed cell lines examined by Nallet-Staub et al., we have extensively characterized TAZ/YAP and Smad crosstalk in numerous cell models, including hESC (Beyer et al., 2013; Varelas et al., 2008), hepatocellular carcinoma cells (Varelas et al., 2008), breast cancer cells (Hiemer et al., 2014), fibroblasts (Liu et al., 2015), and mouse lung development (Mahoney et al., 2014). Other groups described similar crosstalk in mouse embryonic stem cells (Alarcón et al., 2009; Lian et al., 2010), human mesothelioma growth (Fujii et al., 2012), motor neuron differentiation (Sun et al., 2014), regulation of neural stem cells (Yao et al., 2014), bone formation (Yang et al., 2013), endothelial-to-mesenchymal transition of the atrioventricular cushion (Zhang et al., 2014), and skin wound healing (Lee et al., 2014). Thus, while receptor sequestration can occur in epithelial cells, interaction between Hippo and TGFβ signaling is a distinct, robust mechanism that controls Smad signaling and plays a critical role in many important and diverse developmental, homeostatic, and disease contexts.

EXPERIMENTAL PROCEDURES

Cell Culture and Stable Transfection

Eph4 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) as described (Varelas et al., 2010). For HD culture, Eph4 cells were diluted to 5 × 10^5 cells/ml and 0.5 ml or 2.5 ml of cell suspension was plated onto glass coverslips in 24-well plates or 6-well plates, respectively. Cells were serum-starved for 3 hr and stimulated with human TGFβ1 at 100

(D) qRT-PCR. TGFβ-induced target gene expression was assessed in parallel cultures for canonical TGFβ (*Pai1, Smad7*) and TAZ/YAP (*Cyr61*) target genes and are plotted as the mean of the relative expression ± SD (n = 3).

(E) Localization of TGFβ receptor II (TβRII) during cell polarization. Eph4 cells stably expressing TβRII-Clover were cultured for the indicated times, fixed, and costained with phalloidin-Alexa 555. Reconstructed cross-sections are shown.

pM for 1 hr unless otherwise indicated. To generate stable transfectants expressing the TβRII-Clover fusion protein, 5 × 10^5 of Eph4 cells were plated into a 6-well plate and transfected with 2 μg of pCAGIP-TβRII-Clover linearized with PvuI using Lipofectamine LTX (Life Technologies). After 24 hr, cells were trypsinized and plated into 96-well plates with 2 μg/ml puromycin (Sigma-Aldrich) for drug selection. For TGFβ stimulation from the basal side, Transwell inserts (0.4 μm pore size, Corning) were used. Cells were plated according to the surface area of the Transwell insert to acquire either low density (LD, 1:10 dilution of HD) or HD and cultured for 48 hr. Cells were stimulated with human TGFβ1 at 100 pM for 1 hr from the basal side.

Immunofluorescence and Confocal Microscopy

For immunofluorescence, cells grown on a round glass coverslip or Transwell inserts were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature. Cells were washed three times with PBS with 0.1% Tween-20 (PBS-T). For phospho-Smad2/3 staining, cells were permeabilized with 1% SDS in PBS for 10 min at 37°C as described (Varelas et al., 2010). For all other staining, cells were permeabilized with PBS with 0.5% Triton X-100 for 10 min at room temperature. A WAVE FX-X1 spinning disc confocal system (Quorum Technologies) based on a modified Yokogawa CSU-X1 confocal scanner attached to a Zeiss AxioObserver Z1 microscope stand was used for acquiring confocal images from stained samples. A 63×/1.4 or 40×/1.4 plan-apochromat oil immersion objective was used for scanning immunostained samples. Volocity software (Perkin Elmer) was used for image acquisition and processing.

Staining procedures, immunoblotting, and quantitative real-time PCR are described in detail in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.02.019.

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REFERENCES

Alarcon, V.B. (2010). Cell polarity regulator PARD6B is essential for trophectoderm formation in the preimplantation mouse embryo. Biol. Reprod. *83*, 347–358.

Alarcón, C., Zaromytidou, A.I., Xi, Q., Gao, S., Yu, J., Fujisawa, S., Barlas, A., Miller, A.N., Manova-Todorova, K., Macias, M.J., et al. (2009). Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways. Cell *139*, 757–769.

Beyer, T.A., Weiss, A., Khomchuk, Y., Huang, K., Ogunjimi, A.A., Varelas, X., and Wrana, J.L. (2013). Switch enhancers interpret TGF-β and Hippo signaling to control cell fate in human embryonic stem cells. Cell Rep. *5*, 1611–1624.

Fujii, M., Toyoda, T., Nakanishi, H., Yatabe, Y., Sato, A., Matsudaira, Y., Ito, H., Murakami, H., Kondo, Y., Kondo, E., et al. (2012). TGF-β synergizes with defects in the Hippo pathway to stimulate human malignant mesothelioma growth. J. Exp. Med. *209*, 479–494.

Hiemer, S.E., Szymaniak, A.D., and Varelas, X. (2014). The transcriptional regulators TAZ and YAP direct transforming growth factor β-induced tumorigenic phenotypes in breast cancer cells. J. Biol. Chem. *289*, 13461–13474.

Hines, W.C., Su, Y., Kuhn, I., Polyak, K., and Bissell, M.J. (2014). Sorting out the FACS: a devil in the details. Cell Rep. *6*, 779–781.

Lee, M.J., Ran Byun, M., Furutani-Seiki, M., Hong, J.H., and Jung, H.S. (2014). YAP and TAZ regulate skin wound healing. J. Invest. Dermatol. *134*, 518–525.

Lian, I., Kim, J., Okazawa, H., Zhao, J., Zhao, B., Yu, J., Chinnaiyan, A., Israel, M.A., Goldstein, L.S., Abujarour, R., et al. (2010). The role of YAP transcription coactivator in regulating stem cell self-renewal and differentiation. Genes Dev. *24*, 1106–1118.

Liu, F., Lagares, D., Choi, K.M., Stopfer, L., Marinković, A., Vrbanac, V., Probst, C.K., Hiemer, S.E., Sisson, T.H., Horowitz, J.C., et al. (2015). Mechanosignaling through YAP and TAZ drives fibroblast activation and fibrosis. Am. J. Physiol. Lung Cell. Mol. Physiol. *308*, L344–L357.

Mahoney, J.E., Mori, M., Szymaniak, A.D., Varelas, X., and Cardoso, W.V. (2014). The hippo pathway effector Yap controls patterning and differentiation of airway epithelial progenitors. Dev. Cell *30*, 137–150.

Massagué, J. (2012). TGFβ signalling in context. Nat. Rev. Mol. Cell Biol. *13*, 616–630.

Murphy, S.J., Doré, J.J., Edens, M., Coffey, R.J., Barnard, J.A., Mitchell, H., Wilkes, M., and Leof, E.B. (2004). Differential trafficking of transforming growth factor-beta receptors and ligand in polarized epithelial cells. Mol. Biol. Cell *15*, 2853–2862.

Nallet-Staub, F., Yin, X., Gilbert, C., Marsaud, V., Ben Mimoun, S., Javelaud, D., Leof, E.B., and Mauviel, A. (2015). Cell density sensing alters TGF-β signaling in a cell-type-specific manner, independent from Hippo pathway activation. Dev. Cell *32*, this issue, 640–651.

Sun, Y., Yong, K.M., Villa-Diaz, L.G., Zhang, X., Chen, W., Philson, R., Weng, S., Xu, H., Krebsbach, P.H., and Fu, J. (2014). Hippo/YAP-mediated rigidity-dependent motor neuron differentiation of human pluripotent stem cells. Nat. Mater. *13*, 599–604.

Varelas, X., Sakuma, R., Samavarchi-Tehrani, P., Peerani, R., Rao, B.M., Dembowy, J., Yaffe, M.B., Zandstra, P.W., and Wrana, J.L. (2008). TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. Nat. Cell Biol. *10*, 837–848.

Varelas, X., Samavarchi-Tehrani, P., Narimatsu, M., Weiss, A., Cockburn, K., Larsen, B.G., Rossant, J., and Wrana, J.L. (2010). The Crumbs complex couples cell density sensing to Hippo-dependent control of the TGF-β-SMAD pathway. Dev. Cell *19*, 831–844.

Yang, J.Y., Cho, S.W., An, J.H., Jung, J.Y., Kim, S.W., Kim, S.Y., Kim, J.E., and Shin, C.S. (2013). Osteoblast-targeted overexpression of TAZ increases bone mass in vivo. PLoS ONE *8*, e56585.

Yao, M., Wang, Y., Zhang, P., Chen, H., Xu, Z., Jiao, J., and Yuan, Z. (2014). BMP2-SMAD signaling represses the proliferation of embryonic neural stem cells through YAP. J. Neurosci. *34*, 12039–12048.

Zhang, H., von Gise, A., Liu, Q., Hu, T., Tian, X., He, L., Pu, W., Huang, X., He, L., Cai, C.L., et al. (2014). Yap1 is required for endothelial to mesenchymal transition of the atrioventricular cushion. J. Biol. Chem. *289*, 18681–18692.